

Tumor Necrosis Factor Alpha and the Anemia Associated with Murine Malaria

KATHLEEN L. MILLER,* PAUL H. SILVERMAN, BIRGITTA KULLGREN, AND LYNN J. MAHLMANN

*Division of Cell and Molecular Biology, Lawrence Berkeley Laboratory,
University of California, Berkeley, California 94720*

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The anemia associated with malaria is complex, and multiple factors contribute to its severity. An increased destruction and a decreased production of erythrocytes are involved; however, the mechanisms responsible remain unclear. Tumor necrosis factor alpha (TNF- α), released by macrophages in response to infection, is thought to play a role through its ability to inhibit erythropoiesis. In these studies we have examined erythropoiesis in mice infected with *Plasmodium berghei* and in mice infused with recombinant TNF- α via implanted osmotic pumps. In both groups of mice there was (i) a reduction of pluripotent stem cells in the bone marrow and a concomitant increase in the spleen, (ii) a reduction of erythroid progenitor cells, and (iii) a reduced incorporation of ^{59}Fe into erythrocytes. When *P. berghei*-infected mice were given antiserum against recombinant murine TNF, erythropoiesis was partially restored. There was a significant increase in bone marrow stem cells, erythroid progenitor cells, and ^{59}Fe incorporation into erythrocytes in *P. berghei*-infected mice that had been treated with anti-TNF. How TNF may act, directly or indirectly, to inhibit erythropoiesis is not yet clear. These results demonstrate that TNF mediates, in part, the anemia associated with malaria.

The degree of anemia in malaria often cannot be accounted for by the mere removal of parasitized erythrocytes. Rather, the severe anemia of malaria is thought to result from both an increased erythrocyte destruction and a decreased erythrocyte production. The mechanism(s) responsible for the ineffective erythropoiesis is not known; however, evidence suggests an inhibition of the proliferation and differentiation of erythroid progenitor cells or their destruction or both (8, 12, 22, 29, 36). Dyserythropoiesis and reduced numbers of both bone marrow pluripotent stem cells and erythroid precursors have been observed (8, 12, 18, 22, 28, 29, 36). Evidence now suggests that interleukins or cytokines (such as tumor necrosis factor [TNF]) may be involved, rather than direct toxic effects of the parasites (7, 8, 11, 19). TNF alpha (TNF- α), a macrophage-derived cytokine, has been shown to inhibit erythropoiesis in vitro and, more recently, in vivo (1, 6, 25).

Clark has postulated that the activation of macrophages by malarial parasites leads to excessive production of TNF, which mediates much of the pathology associated with malaria through its action on a variety of cell types (7, 11). Grau et al. have shown that the cerebral malaria in CBA/Ca mice infected with *Plasmodium berghei* anka can be abrogated if the mice are treated with anti-TNF antiserum (14). TNF has also been shown to play a role in the early fetal death and abortion observed with murine malaria (9). In addition, Clark and Chaudhri observed an increase in erythrophagocytosis and dyserythropoiesis in the bone marrow of *P. vinckei*-infected mice given recombinant human TNF (rHuTNF) (8).

In this paper we extend our studies of the effects of malaria infection on hemopoiesis and examine the effects of TNF on both pluripotent stem cells and erythropoiesis. We demonstrate that TNF may play a role in the anemia of malaria through its ability to inhibit erythropoiesis.

MATERIALS AND METHODS

Mice. Female BALB/c mice (Bantin and Kingman, Fremont, Calif., and Simonsen, Gilroy, Calif.) between 2 and 3 months of age were used and maintained on standard mouse chow and water ad libitum.

Parasites and infection of mice. *P. berghei* was originally obtained from A. C. Allison and E. M. Eugui, Syntex, Palo Alto, Calif. Mice were inoculated intravenously with 10^5 parasitized erythrocytes, and parasitemias were determined by examining 200 to 1,000 erythrocytes from tail blood smears stained with Dif Quik (American Scientific Products, McGaw Park, Ill.). Parasites were maintained by serial passage of 10^5 parasitized erythrocytes when the parasitemia was rising. After 10 passages, a fresh inoculum was initiated from stocks frozen in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) and stored in liquid nitrogen. Parasites were passaged at least once before experimental use.

Hematological parameters. Blood was obtained by cardiac puncture with heparin sodium (The Upjohn Co., Kalamazoo, Mich.) as an anticoagulant. Hematocrits were determined by using a microcentrifuge (Damon/IEC, Needham Heights, Mass.). Reticulocytes were counted by light microscopy after staining them with new methylene blue (J. T. Baker Chemical Co., Phillipsburg, N.J.) (5). Cell counts were done in Isoton (Curtin Matheson Scientific, Inc., Houston, Tex.) with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.), and lyzerglobin (Baker Instrument Co., Allentown, Pa.) was added for nucleated cell counts. For incorporation of ^{59}Fe , mice were injected intravenously with 0.5 μCi of ^{59}Fe in mouse plasma ($^{59}\text{FeCl}_3$, 12 to 20 mCi/mg of Fe; Dupont, NEN Research Products, Boston, Mass.) and sacrificed 6 h later, and tissues were removed for counting in a Packard gamma counter (Packard Instruments, Downers Grove, Ill.).

Cell suspensions. Single-cell suspensions of bone marrow or spleen were made in RPMI 1640 supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesul-

* Corresponding author.

fonic acid) buffer (GIBCO) and 50 μ g of gentamicin sulfate (Sigma) per ml and filtered through sterile 150- μ m spectra mesh filters (Spectrum Medical Industries, Inc., Los Angeles, Calif.). Cell counts were done as described above, and cell viability was determined by eosin exclusion.

Assays for BFU-E and CFU-E. Burst forming units-erythroid (BFU-E) and colony forming units-erythroid (CFU-E) were determined by culture in methylcellulose by using a modification of the method of Stewart et al. (30). The medium consisted of 0.8% methylcellulose (Dow Chemical, Walnut Creek, Calif.), 1 mM L-glutamine (GIBCO), 10^{-4} M mercaptoethanol (Sigma), 1% detoxified bovine serum albumin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 30% fetal bovine serum (Flow, Inglewood, Calif.), 10^{-4} M hemin (Sigma), and 50 μ g of gentamicin sulfate (Sigma) per ml in Iscove modified Dulbecco medium (GIBCO). Recombinant human erythropoietin (70,000 IU/mg; AmGen, Thousand Oaks, Calif.) was used at a final concentration of 1 U/ml for BFU-E and 0.5 U/ml for CFU-E. Cultures were incubated in 5% CO₂-5% O₂-90% N₂ in saturated humidity and scored unstained. After 48 h, aggregates of 8 to 64 hemoglobinized cells were scored as CFU-E. After 7 days, clusters of three or more aggregates containing 20 or more hemoglobinized cells were scored as BFU-E.

Assay for CFU-S. The standard method of Till and McCulloch (34) for determining pluripotent stem cells (CFU-S) was used. Nine-day spleens were fixed in Tellyesniczky solution, and spleen colonies were counted at a magnification of $10\times$. Before transplantation, the recipient mice were irradiated with 730 cGy by using a ⁶⁰Co source. At this dose the irradiated control mice had less than 1 CFU-S per spleen.

TNF and antisera. rHuTNF- α was kindly supplied by Genentech Inc., South San Francisco, Calif. (specific activity, 6.72×10^7 U/mg; activity, 1 endotoxin unit per ml in the *Limulus* amoebocyte lysate assay). Recombinant murine TNF- α (rMuTNF- α), produced by Suntory of Japan, was kindly supplied by Biogen, Cambridge, Mass. (specific activity, 2×10^7 to 4×10^7 U/mg). Aliquots of TNF were stored at -70°C until use. Once thawed and further diluted, they were kept at 4°C for up to 2 weeks. Polyclonal rabbit antiserum to rMuTNF was kindly supplied by Biogen (specific activity, 10^4 neutralizing units per ml).

Implantation of osmotic pumps. Osmotic pumps that contained ca. 200 μ l and released approximately 1 μ l/h for 7 days (Alzet, Palo Alto, Calif.) were aseptically implanted under the dorsal skin of mice under methoxyflurane (Pitman Moore, Washington Crossing, N.J.) anesthesia, and the wound was closed with surgical clips.

Statistics. The significance of differences between mean values were analyzed by using the Student's two-tailed *t* test. Only cases with $P < 0.05$ were reported as significant. In addition, the Wilcoxon matched-pairs signed-rank test was used to determine the significance of differences. Two-tailed *P* values of <0.05 were reported as significantly different.

RESULTS

Erythroid progenitor cells in the bone marrow and spleens of *P. berghei*-infected mice. To characterize the effects of malaria infection on erythropoiesis, the relative numbers of early erythroid progenitor cells in the bone marrow and spleens of infected mice were determined. Mice were infected with *P. berghei*, and at various days postinfection the bone marrow and spleens were assayed for CFU-E and BFU-E. The results are shown in Fig. 1. Normal levels of the

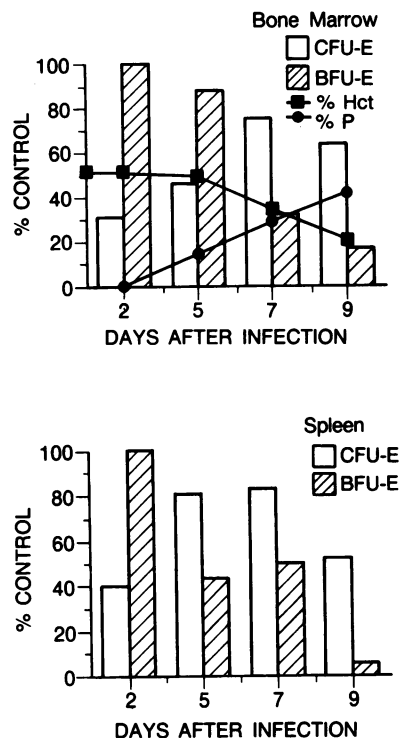


FIG. 1. Relative numbers of early erythroid progenitor cells (BFU-E and CFU-E) in the bone marrow and spleen of mice over the course of infection with *P. berghei*. BFU-E and CFU-E were determined from cells pooled from groups of three mice by using in vitro colony assays. Data are expressed as the percentage of the mean of control values obtained for normal mice. The mean values \pm standard error in normal mice were: CFU-E per femur, $5.65 \times 10^4 \pm 0.56$; BFU-E per femur, $4.26 \times 10^3 \pm 0.26$; CFU-E per spleen, $5.17 \times 10^4 \pm 1.19$; BFU-E per spleen, $7.81 \times 10^3 \pm 1.19$. % P, % Parasitemia; % Hct, % hematocrit.

less mature BFU-E were present in both the spleen and bone marrow on day 2 of infection and then declined over the course of infection as compared with in normal mice. By day 7, there was a significant reduction in BFU-E in both bone marrow (36% of control; $P < 0.01$) and spleens (50% of control; $P < 0.05$) of infected mice. In contrast, the more mature CFU-E were present in reduced numbers in both bone marrow (31% of control; $P < 0.01$) and spleens (40% of control; $P < 0.01$) as early as 2 days postinfection. This was followed by a transient increase in the number of CFU-E followed by a decline 9 days postinfection. This transient increase was probably in response to the increased levels of circulating serum erythropoietin that we and others have observed at this time in infected mice (24, 28). Similar results were obtained in another experiment, except that CFU-E levels, particularly in the spleen, rose to well above normal levels but then declined to below normal levels by day 7 postinfection, as discussed above.

Effects of rHuTNF on pluripotent stem cells (CFU-S) in normal mice. To compare the effects of malaria and TNF on hemopoiesis, early studies focused on the effects of TNF on pluripotent stem cells (CFU-S) in normal mice. Due to the reported short half-life (30 min) of TNF, osmotic pumps were used to deliver sustained levels to mice (3). Pumps containing rHuTNF were implanted into normal mice, and 6 days later the number of CFU-S in the bone marrow, spleen, and blood were determined. The pumps released approxi-

TABLE 1. Effects of rHuTNF on the CFU-S contents of bone marrow, spleens, and blood of normal mice^a

Cell type and treatment	10 ⁶ Nucleated cells/organ (mean ± SEM)	CFU-S/10 ⁶ cells (mean ± SEM)	CFU-S/organ (mean ± SEM)
Bone marrow			
rHuTNF	11.0 ± 0.41	190 ± 14 ^b	2,100 ± 130 ^c
RPMI 1640	13.6 ± 0.95	280 ± 23	3,800 ± 160
Spleen			
rHuTNF	130 ± 20	170 ± 14 ^d	27,000 ± 2,700 ^d
RPMI 1640	170 ± 3.5	20 ± 1	3,500 ± 200
Blood			
rHuTNF	22.0 ± 5.1 ^{c,e}	24 ± 6 ^d	520 ± 53 ^{d,f}
RPMI 1640	9.7 ± 1.4 ^c	1 ± 0.3	11 ± 1.9 ^f

^a Experimental mice received approx 1.8×10^5 U of rHuTNF (Genentech) per day for 6 days via implanted osmotic pumps. Control mice received RPMI 1640. Groups were of seven mice each.

^b $P < 0.01$ compared with RPMI 1640 group by Student's *t* test.

^c $P < 0.05$ compared with RPMI 1640 group by Student's *t* test.

^d $P < 0.001$ compared with RPMI 1640 group by Student's *t* test.

^e Cells per milliliter of blood.

^f CFU-S per milliliter of blood.

mately 1.8×10^5 U/day, equivalent to 2.73 µg of rHuTNF per day. Osmotic pumps in control mice delivered RPMI 1640. The results of three separate experiments were combined and appear in Table 1. Mice that had been infused with rHuTNF showed a significant ($P < 0.05$) reduction in bone marrow CFU-S and a significant ($P < 0.001$) increase in spleen and blood CFU-S as compared with control mice. These results are similar to those observed by us earlier for *Plasmodium*-infected mice (28).

Effects of rMuTNF on hemopoiesis in normal mice. Studies on the effects of TNF on hemopoiesis in normal mice were extended to include its effects on erythropoiesis, as well as on pluripotent stem cells. In addition, rMuTNF was used as it became available. Osmotic pumps containing rMuTNF were implanted into groups of six mice. Seven days later the mice were sacrificed and the number of CFU-S, BFU-E, and CFU-E in the bone marrow and spleen were determined. The pumps released approximately 10^5 U of rMuTNF per day, equivalent to 2.8 µg/day. Osmotic pumps in control mice delivered RPMI 1640. The combined results of two experiments appear in Table 2. As with rHuTNF, infusion with rMuTNF resulted in significant ($P < 0.05$) depletion of bone marrow stem cells (CFU-S) and a concomitant significant ($P < 0.01$) increase in the spleen. Mice infused with

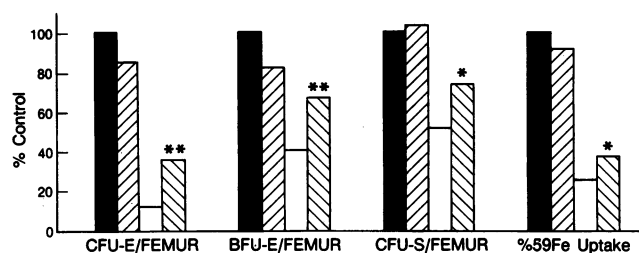


FIG. 2. Effects of antisera to TNF on hemopoiesis in *P. berghei*-infected mice. Groups of five normal or *P. berghei*-infected mice were given 2×10^5 neutralizing units of a polyclonal rabbit antiserum to rMuTNF (anti-TNF) 1 day preinfection and 3 days postinfection. Controls were given NRS. Six days postinfection, mice were sacrificed and the numbers of BFU-E, CFU-E, and CFU-S per femur were determined. In addition, the percentage of ⁵⁹Fe incorporation per femur (%59Fe uptake) was also determined. The combined data from three experiments are expressed as the percentage of the mean of control values obtained for normal mice given NRS. ■, Normal uninfected mice plus NRS; ▨, normal uninfected mice plus anti-TNF; □, *P. berghei*-infected mice plus NRS; ▩, *P. berghei*-infected mice plus anti-TNF. *, $P < 0.03$; **, $P < 0.01$ (Wilcoxon signed-rank test).

rMuTNF for 7 days did not show evidence of anemia; erythrocytes per millimeter³ of blood, reticulocytes per millimeter³ of blood, and hematocrit (%) were not significantly different from those of control mice (data not shown). Mice treated with TNF did show a significant reduction in the number of CFU-E ($P < 0.05$) and BFU-E ($P < 0.01$) in the bone marrow and CFU-E ($P < 0.05$) and BFU-E ($P < 0.05$) in the spleen.

Effects of antisera to rMuTNF on hemopoiesis in *P. berghei*-infected mice. To determine whether TNF is involved in the ineffective erythropoiesis observed in infected mice, *P. berghei*-infected mice were given antiserum to rMuTNF. Groups of five normal mice were injected intraperitoneally with 2×10^5 neutralizing units of polyclonal rabbit antiserum against rMuTNF (anti-rMuTNF) per mouse or 0.2 ml of normal rabbit serum (NRS) per mouse as a control. The next day, groups of five treated mice were infected with *P. berghei*. Groups of normal or infected mice received a second injection of anti-rMuTNF or NRS 2 days later. Mice were sacrificed on day 6 postinfection, and the number of CFU-S, BFU-E, and CFU-E in the bone marrow and spleen were determined along with ⁵⁹Fe incorporation into erythrocytes. The combined results of three separate experiments

TABLE 2. Effects of rMuTNF on hemopoiesis of normal mice^a

Cell type and treatment	10 ⁶ Cells/femur or spleen (mean ± SEM)	CFU-S/femur or spleen (mean ± SEM)	CFU-E/femur or spleen (mean ± SEM)	BFU-E/femur or spleen (mean ± SEM)
Bone marrow				
rMuTNF	15 ± 1.1 ^b	2,220 ± 119 ^c	67,309 ± 4,007 ^c	915 ± 298 ^d
RPMI 1640	17 ± 1.2 ^b	4,112 ± 290	87,253 ± 5,667	2,639 ± 261
Spleen				
rMuTNF	159 ± 9 ^b	47,343 ± 1,391 ^d	28,653 ± 1,017 ^c	8,680 ± 358 ^c
RPMI 1640	132 ± 11 ^b	3,986 ± 1,031	45,768 ± 893	14,768 ± 218

^a Experimental mice received approximately 10^5 U of rMuTNF (Biogen) per day for 7 days via implanted osmotic pumps. Control mice received RPMI 1640. Results are from three experiments with groups of six mice each.

^b Nucleated cells.

^c $P < 0.05$ compared with RPMI 1640 group by Student's *t* test.

^d $P < 0.01$ compared with RPMI 1640 group by Student's *t* test.

are shown in Fig. 2. Mice infected with *P. berghei* for 6 days and given NRS as a control had a significant anemia as evidenced by a reduction in hematocrits ($P < 0.01$) and erythrocytes per millimeter³ of blood ($P < 0.01$) (data not shown). In addition, the numbers of CFU-E ($P < 0.001$), BFU-E ($P < 0.05$), and CFU-S ($P < 0.01$) were significantly reduced in the bone marrow (Fig. 2). The incorporation of ⁵⁹Fe was also reduced in both the bone marrow ($P < 0.01$) (Fig. 2) and spleen ($P < 0.05$) (data not shown) in infected mice. In the spleen, CFU-S were significantly ($P < 0.01$) increased, while CFU-E and BFU-E were significantly reduced ($P < 0.01$) (data not shown). These results are in agreement with those reported earlier for mice infected with *P. berghei*.

When *P. berghei*-infected mice were given anti-rMuTNF they showed a significant increase in the number of bone marrow CFU-E ($P < 0.01$), BFU-E ($P < 0.05$), and CFU-S ($P < 0.05$) and in ⁵⁹Fe incorporation ($P < 0.05$) as compared with *P. berghei*-infected mice given NRS (Fig. 2). In contrast to the bone marrow, there were significant changes only in CFU-E (increased [$P < 0.01$]) and CFU-S (decreased [$P < 0.05$]) in the spleens of infected mice given anti-rMuTNF (data not shown). These results demonstrated that treatment with antiserum against rMuTNF can prevent some of the ineffective erythropoiesis observed in *P. berghei*-infected mice.

DISCUSSION

Our investigations of the effects of malaria on hemopoiesis focused, initially, on its effects on pluripotent stem cells (CFU-S). Studies by Silverman et al. (28) in our laboratory showed that during the course of infection with *P. berghei*, *P. vinckei*, or *P. chabaudi adami* there is a significant depletion of the pluripotent stem cells in the bone marrow followed by a concomitant increase in the spleen. The increase in spleen stem cells, however, does not appear to compensate for the decreased erythropoietic capacity of the bone marrow. In the studies reported here, we have shown that normal mice infused with rHuTNF or rMuTNF via osmotic pumps show a similar decrease in pluripotent stem cells (CFU-S) in the bone marrow and an increase in these cells in the spleen. In addition, the depletion of bone marrow stem cells observed in *P. berghei*-infected mice was significantly reduced if the mice were given anti-rMuTNF. A decrease in bone marrow cellularity and CFU-C in mice given TNF intravenously has been reported by Talmadge et al. (32). The alterations in stem cells observed in cases of malaria are probably the result of the migration of stem cells from the bone marrow to meet the erythropoietic demands of the anemia (23). The changes in stem cells also appear to involve, in part, the direct or indirect action of TNF. Stem cell depletion in the bone marrow of infected mice occurs before detectable anemia (28) and can be partially prevented by treatment with anti-rMuTNF.

Malaria is also associated with alterations in erythropoiesis. Over the course of infection with *P. berghei* there was an overall decline in erythroid progenitor cells (CFU-E and BFU-E) in the bone marrow and spleen. For instance, as early as day 2 postinfection CFU-E were present in decreased numbers and, despite a transient rise, probably in response to increasing levels of erythropoietin, declined to below normal levels. Maggio-Price et al. showed that BFU-E were depleted in the bone marrow throughout *P. berghei* infection in C57BL/6J mice. After a decline during the first week of infection, CFU-E were present in increased num-

bers (18). These results suggest that despite the presence of significant levels of erythropoietin and an increased demand for erythrocytes, *P. berghei*-infected mice cannot sustain erythropoiesis. The decreased production of erythrocytes in malaria may be the result of events inhibiting the differentiation or proliferation or both of erythroid precursors or perhaps their differentiation from stem cells.

TNF- α , which is produced during host responses to microbial infections, is a major mediator of the inflammatory response and exerts a wide array of biological activities on a variety of cell types (4, 21). It is a potent inhibitor of erythropoiesis both in vitro and in vivo (1, 6, 25). The side effects associated with infusion of high amounts of TNF are very similar to the pathophysiology of clinical malaria (7, 11, 16, 26), and increased TNF production has been observed in cases of both human and murine malaria (8, 14, 27). Clark has proposed that much of the pathology of malaria, including anemia, could be mediated by TNF (7, 11). In addition to pathological consequences, TNF appears to also have protective effects and has been shown to inhibit the multiplication of malarial parasites in vivo (10, 33). Thus, low levels of TNF may be protective while excessive production can be harmful.

In these studies, we have shown that mice infused in vivo with rMuTNF have reduced numbers of erythroid progenitors (CFU-E and BFU-E) in the bone marrow and spleen. We did not observe a significant anemia in mice infused for 1 week with rMuTNF or rHuTNF. One week may not have been sufficient time for the development of detectable anemia, considering the 52-day lifespan of mouse erythrocytes (17). Johnson et al. reported a significant anemia after 3 weeks in nude mice transplanted with CHO cells constitutively expressing the human TNF gene (R. A. Johnson, T. A. Waddelow, A. Oliff, and G. D. Roodman, Clin. Res. 36:412, 1988). Clark and Chaudhri have reported that appreciable erythrophagocytosis and dyserythropoiesis were observed in mice only if they had been primed with extracts of *Coxiella burnetii* or low-level infections with *P. vinckei* before receiving TNF (8). Mice infected with *Plasmodium* species or presensitized with bacteria are apparently much more susceptible to the effects of TNF. In human malaria, anemia is often more severe in patients with concomitant bacterial infections (22).

The anemia of malaria is complex and both an increased destruction and a decreased production of erythrocytes contribute to the degree of anemia. The ability to partially restore erythropoiesis in *P. berghei*-infected mice by treatment with anti-TNF antiserum demonstrates that the ineffective erythropoiesis observed in these mice is mediated in part by TNF. When *P. berghei*-infected mice were given anti-rMuTNF, erythroid precursors (CFU-E and BFU-E) in the bone marrow and CFU-E in the spleen were significantly increased as compared with infected control mice given NRS. In addition, ⁵⁹Fe incorporation in the bone marrow was also significantly increased. It is not yet clear whether TNF acts directly or indirectly to inhibit erythropoiesis. TNF may play a role through its demonstrated ability to inhibit erythroid precursors (1, 6, 25) or indirectly by stimulating the release of tissue-damaging reactive oxygen substances (2, 10, 11, 16, 26). It may also play a role by enhancing monocyte functions such as erythrophagocytosis (8) or promoting monocyte differentiation at the expense of erythrocytes or both. Competition among stem cell lineages has been postulated to occur in malaria (13). TNF has been shown to be an autocrine for the monocytic lineage (31, 35) and to stimulate the release of macrophage colony-stimu-

lating factor (15, 20). Whatever the mechanism(s), these studies have shown that TNF plays a role, in part, in mediating the anemia of malaria.

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